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Monitoring of unfolding and refolding in fungal phytase (phyA) by dynamic light scattering

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Abstract

Role of disulfide bridges in phytase's unfolding–refolding was probed using dynamic light scattering. Phytase was unfolded by guanidinium chloride and then refolded by removing the denaturant by dialysis. Thiol reagents prevented refolding; thus, disulfide bridge formation is an integral step in phytase folding. Catalytic demise of phytase after unfolding and refolding in presence of Tris(2-carboxyethyl)phosphine (TCEP) indicates that disulfide bridges are necessary for refolding. The hydrodynamic radius (rh) of active and unfolded phytase is 4 and 14 nm, respectively. Removal of denaturant through dialysis refolds phytase; its rh shifts back to 4 nm. When TCEP remains in the refolding media, the rh remains high. The unfolded phytase when diluted in assay medium refolds as a function of time at 25 and 37 °C, but not at higher temperature. Monitoring rh under denaturing and renaturing condition gives an accurate measure of the folding status of phytase.
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Biochemists are increasingly studying protein folding, unfolding, and refolding mechanisms to have an insight into how proteins' structure dictates stability. Secreted fungal enzymes such as phytase are very stable proteins [1]. Therefore, a study of unfolding and refolding of fungal phytase would shed light on the structural stability in this class of proteins. *Myo*-inositol hexakisphosphate phosphohydrolase, phytase, belongs to 'histidine acid phosphate' or HAP, an important subclass of acid phosphates that had attracted attention both from academic and industrial researchers [2]. One important member of this group, the fungal phyA, is the focus of study in our laboratory since 1985. We purified and characterized this enzyme in 1987 [1], cloned the gene encoding the enzyme in 1991 [3], identified the active site [4], and sequenced the protein by micro Edman method in 1993 [5]. Since then, many laboratories worldwide have turned their focus on this enzyme [6]. The enzyme's three-dimensional structure has already been deduced by X-ray crystallography [7]. The fungal phytase exhibits a compact β-strand dominated lower domain and a loose alternating α/β rich domain on the upper portion; a donut-shaped active site is located in the interface of these two domains. Recently, knowledge-based site-directed mutagenesis in the substrate-binding domain had resulted in shifting the pH optima of the enzyme that will favor catalysis in the stomach of monogastric animals such as poultry [8]. The future looks very promising to tailor-make the enzyme for diversified uses among which lowering phytate content of animal's fecal matter is of prime consideration. Undigested phytate present in soybean meal due to lack of active phytase in animals' gut creates environmental problems. The fecal material contains high phytate that may favor microbial growth and concomitant release of free phosphate due to abundance of microbial phytase produced by

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bacteria and fungi. The released phosphates may contaminate groundwater that end up in large bodies of water. The excess nutrients, especially phosphate, induce harmful algal bloom resulting in red zone and fish kill [9]. To combat this problem, the State of Maryland recommended that phytase be used as a supplement to poultry and hog diets.

Commercialization of fungal phytase had ensued in the late 1990s. Two companies have cloned and overproduced two native phytases, one by *Aspergillus ficuum*, and the other by a basidiomycete, *Peniophora lycii*. We recently compared the two commercial phytases, Natuphos and Ronozyme, for stability, catalysis, and physico-chemical properties [10]. Based on the results we concluded that even though *Peniophora lycii* phytase is endowed with higher catalytic activity, it lacks the stability and optimum pH of the phytase produced by ascomycete fungus, *A. ficuum*. Recently, we have performed site-directed mutagenesis in the substrate-binding domain of *Aspergillus ficuum* phytase to increase catalytic activity about fourfold (unpublished data).

Both the stability in *A. ficuum* phytase and its ability to perform optimally at 58 °C are attributed to the presence of five disulfide bridges [2]. Previously, we have shown that disulfide bridge formation is a prerequisite to folding in *A. ficuum* phytase [11]. Recent thermal denaturation studies with fungal enzyme exhibiting both phytase and acid phosphatase have shown that the rapid inactivation and slow conformational changes took place during heat-induced denaturation; inactivation had occurred before structural alteration could take place [12].

In this paper, we present additional data to support that disulfide bridge formation, all five of them, is very important for folding. When unfolded phytase is treated with Tris(2-carboxyethyl)phosphine (TCEP), a thiol reagent, the enzyme did not fold properly. Consequently, no phosphohydrolase activity can be detected in the refolded protein. Also, protein aggregates were formed as evidenced by a higher value for the hydrodynamic radius (rh) of both the unfolded and refolded phytase in presence of TCEP. The refolded phytase however show a lower rh value of about 4.1 nm. The unfolded phytase forms aggregate, which gives a higher rh value. Therefore, dynamic light scattering instrument can be used to study protein folding, unfolding, and refolding.

Materials and methods

Source of phytase. *Aspergillus ficuum* (*niger*) phytase was obtained from Gist-brocade/BASF (Delft, The Netherlands), which is the recombinant product of *A. ficuum phyA* gene. We have purified this enzyme using Macro-Prep S and Q columns. The purified phytase had approximate specific activity of 3000 nKats/mg protein and showed a single band on SDS–PAGE.

Sample preparation. The purified phytase preparations were dialyzed against 25 mM sodium acetate, 125 mM sodium chloride, pH 5.0, buffer in order to remove glycerol, which could interfere with dynamic light scattering measurements. This glycerol free sample was also used for monitoring enzyme activity.

Phytase assay. Phytase assays were carried out in 1.0 mL volume at 58 °C in 50 mM sodium acetate (assay buffer) at pH 5.0 similar to *A. ficuum* phytase assay [2]. The liberated inorganic *ortho*-phosphates were quantitated spectrophotometrically using a freshly prepared Acetone–acid–molybdate (AAM) reagent consisting of acetone, 10 mM ammonium molybdate, and 2.5 M sulfuric acid (2:1:1, v/v). Adding 2.0 mL AAM solution per assay tube terminated phytase assay. After 30 s, 0.1 mL of 1.0 M citric acid was added to each tube. Absorbance was read at 355 nm after blanking the spectrophotometer with appropriate control. Values were expressed as nKats/mL. Kat stands for katal, the amount of enzyme that converts one mole of substrate to product per second.

Acid phosphatase assay. The phytase samples, typically 40–50 μL having a concentration of 1 mg/mL, in the above assay buffer were incubated with 1.25 mmol *p*-nitrophenol phosphate (pNPP) in a final volume of 1.0 mL at 58 °C for 1 min. The reaction was terminated using 0.1 mL of 1.0 M NaOH and the liberated *p*-nitrophenol was measured at 400 nm.

Dynamic light scattering measurements. The phyA samples under unfolding and refolding conditions were subjected to dynamic light scattering measurements using PDDLS/Batch Instrument (Precision Detectors, Bellingham, MA 02019). The software PrecisionDeconvolve (ver 2.1) was run to collect, process, and store light scattering data. The measurements were made at ambient temperature (29 °C), run time 4 (ms), and smoothness 8. Using Precision DeconView rh values were obtained from the size distribution plot. The values represent minimum of 3–5 measurements of the same sample.

Unfolding of the phytase. To a 500 μL of the phytase in assay buffer was mixed GuCl (Pierce, Rockford, IL) to achieve a concentration of 8.0 M. The reaction mix was then incubated at room temperature to achieve complete inactivation. The above ratio between protein and salt was always maintained during all the unfolding experiments.

Refolding of phytase. The phytase enzyme refolded when diluted or dialyzed against the assay buffer. To facilitate refolding, the GuCl was removed either by dialysis or by buffer exchange using Centricon (Amicon) concentrator. Similar experiments were done in presence of reducing agents such as β-mercaptoethanol (β-ME), 25 mM, or TCEP (20 mM).

Time course studies of phytase unfolding. An aliquot (12 μL) of the GuCl-treated phytase was mixed with 913 μL assay buffer and kept at 22, 37, and 58 °C between 0 and 120 min. Phytase assay was done at 58 °C for 1 min after adding 75 μL of 10 mM phytate.

Effect of temperature on the phytase unfolding. Twelve microliters of phytase A was mixed with 913 mL assay buffer and kept for 5 min at 22–47 °C and phytase activity was measured at 58 °C as before.

Results

The recombinant phytase produced in an industrial strain of *Aspergillus niger* by Gist-brocade, which is marketed as Natuphos, was used in the unfolding–refolding studies. We purified the crude phytase to near homogeneity by sequential ion-exchange chromatographies. Fig. 1 shows the level of purity as judged by SDS–PAGE. As can be seen, the protein fraction that we used is nearly homogeneous with an approximate size of 85–100 kDa.

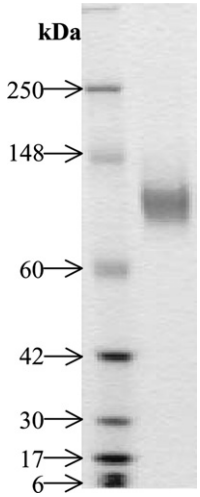

Fig. 1. SDS–PAGE profile of the purified *A. ficuum* phytase.

Critical disulfide bridges in phytase

The primary sequence of *A. ficuum* phytase is shown in Fig. 2. As can be seen, 10 cysteine residues participate in forming 5 disulfide bridges. The pairings of cysteine residues were obtained from the three-dimensional structure of phytase as deduced by X-ray crystallographic method [7]. Of all five disulfide bridges, only two, pair number 2 and 3, are critical because these cysteine residues are conserved among 'HAP' proteins (data not shown). The cysteine residues forming first disulfide bridge ① are not present in phyB type of fungal phytase. The other two disulfide bridges may play a role in the stabilization of the molecule. What role does these disulfide bridges play in the folding pathway of fungal phytase is conjectural at this point.

Unfolding of phytase and reduction of disulfide bonds by β-ME

Unfolding of phytase was achieved by GuCl at a concentration of 8.0 M. β-ME (25 mM) was used to reduce the disulfide bridges of the protein. Table 1 summarizes the results. When dialysis was performed in the presence and absence of the reducing agent (β-ME), the protein was refolded partially. More activity was achieved in the preparation that did not have the reducing agent. It is possible that the cysteine residues were re-oxidized upon dialysis.

Unfolding of phytase and reduction of disulfide bonds by TCEP

To evaluate the effect of a potent reducing agent while phytase had unfolded, we used TCEP. The experiments performed were near identical to the one described earlier but with two variations. First, TCEP was used as thiol protecting reagent. Second, Centricon was used to dialyze the denaturant, GuCl. Results of unfolding and refolding in the presence and absence of TCEP are shown in Table 2. TCEP prevented oxidation of cysteine residues. Therefore, disulfide bridge formation in phytase was prevented. Consequently, the proteins were not folded properly. Dialysis by Centricon did not introduce molecular oxygen into the reaction mix as compared to rapid dialysis method. Thus, cysteine residues remain in reduced state; thereby, disulfide bridges were not formed. This had dire consequence on phytase refolding. The protein did not refold into an "active form." The catalytic demise due to addition of GuCl cannot be reversed by dialysis performed by Centricon in the presence of TCEP. In the control experiment, the GuCl-treated proteins had not encountered a problem refolding upon dialysis. The problem of

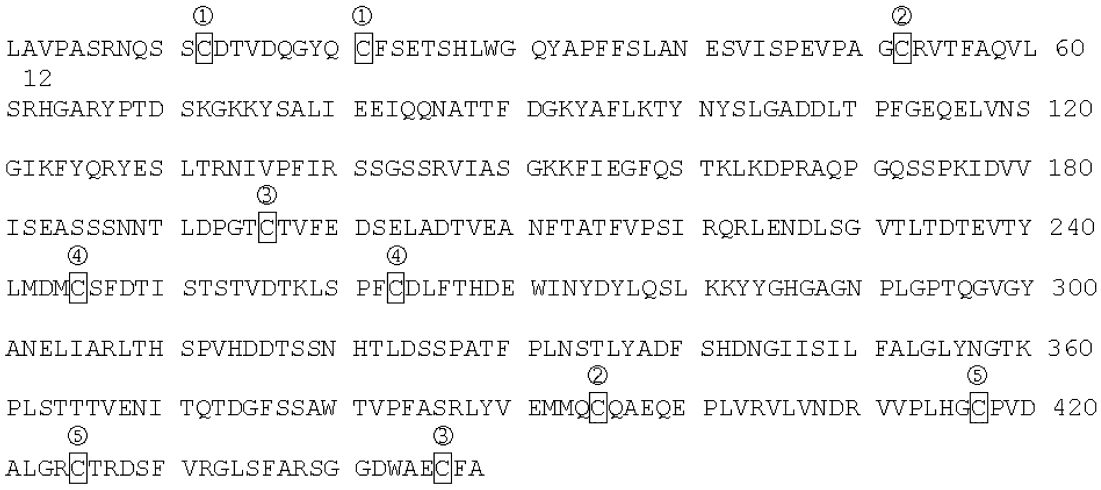

Fig. 2. The primary sequence of *A. ficuum* phytase showing pairings of five disulfide bridges as deduced from the three-dimensional X-ray crystallography [7] of fungal phytase from the same species.

Table 1
Unfolding and reduction of the disulfide bonds using 25 mM β-mercaptoethanol

Substrate	Control		GuCl treated[a]		GuCl and β-ME[b]	
	nKats/mL	%	nKats/mL	%	nKats/mL	%
Phytate						
Before dialysis	2329	100.0	22	0.6	22	0.6
After dialysis	2300	98.0	1825	78.0	1507	68.0
p-NPP						
Before dialysis	167	100.0	0	0.6	0	0.6
After dialysis	167	100.0	162	97.0	116	69.0

[a] Fifty millimolar acetate buffer, pH 5.0, was used as a dialysis buffer.
[b] Fifty millimolar acetate buffer, pH 5.0, containing 25 mM β-ME was used as a dialysis buffer.

Table 2
Effect of reducing agents on the phytase A folding and unfolding states

Unfolding/refolding conditions	Control		GuCl treated[a]		GuCl + β-ME/TCEP[b]	
	nKats/mL	%	nKats/mL	%	nKats/mL	%
β-ME as a reducing agent						
Before buffer change using Amicon	3582	100.0	40	1.1	40	1.10
After buffer change using Amicon	3582	100.0	3412	95.0	855	24.00
TCEP as a reducing agent						
Before buffer change using Amicon	3656	100.0	119	3.3	102	2.70
After buffer change using Amicon	3639	99.5	3469	95.0	107	2.90

[a] During buffer exchange 50 mM acetate, pH 5.0, maintained.
[b] During buffer exchange the above buffer had either 25 mM β-ME or 20 mM TCEP.

refolding only arose when disulfide bridge was not allowed to be formed by the incorporation of TCEP in the reaction mix (Table 2).

The rh values of phytase during unfolding and refolding

Hydrodynamic radius (rh) of a protein may shed light on the overall structure of a protein molecule. For example, the native phytase, which is catalytically active, gives an rh value of about 4 nm [10]. A larger rh value indicates aggregation of proteins. We used this simple concept to probe the rh values of fungal phytase as both unfolding and refolding processes are taking place. Table 3 summarizes the data. As expected, monomeric form of the native and functional phytase assumes a shape that gives an rh value of about 4 nm. However, the denaturant-induced unfolded phytase gives a much higher value (13–14 nm). The aggregated proteins give a molecular weight of 1.5–18 million Da (Table 3). When the denaturant is removed by rapid dialysis either in the presence or in absence of thiol active reagent, β-ME, refolding takes place and the protein assumes an rh value of about 4 nm. The refolded phytase regains catalytic activity. Therefore, it follows that refolding brings down the rh value from about 14 to 4 nm, which results in the regeneration of active site. When thiol active reagent, TCEP, is maintained during the removal of denaturant, GuCl, by Centricon, the denatured phytase assumes a very high rh value (about 13 nm) with almost a total loss of activity (Table 3). When the same denatured preparation is placed in Centricon to remove the denaturant in the absence of any thiol reagent or in the presence of β-ME, phytase activity is partially restored and the protein assumes an intermediate rh value (about 5–6.5 nm).

Effect of temperature on refolding of unfolded phytase

When the denaturant, GuCl, is introduced into fungal phytase at a concentration of 1 M, the protein is unfolded rapidly [11]. However, if the denatured (unfolded) phytase is diluted by dispensing into the assay medium, phytase activity is recovered as a function of time (Fig. 3). However, this is a temperature dependent phenomenon. Refolding of phytase due to dilution of denaturant concentration in assay medium is not an issue at temperature 22–37 °C. However, at a temperature beyond 40 °C, the unfolded protein never refolds correctly to assume an optimal structure to engender an active site (Fig. 3). However, we have not examined what happens to the rh value of phytase at these temperatures because of the limitation of the instrument Fig. 4.

Discussion

The role of disulfide bridges in unfolding and refolding of *A. ficuum* phytase has been probed by GuCl.

Table 3
Correlation between phytase activity and rh values during unfolding and refolding

Phytase A enzyme (unfolded, refolded states)	Phytase activity (%)	rh (nm)
Control	100.0	4.09
GuCl treated	1.1	13.20
GuCl + 25 mM β-ME	1.1	14.40
Dialysis (to remove GuCl)		
Control (not reacted with GuCl)	100.0	4.09
GuCl removed	98.0	4.10
GuCl removed in the presence of 25 mM β-ME	78.0	4.40
Centricon (to remove GuCl)		
Control (not reacted with GuCl)	100.0	3.96
GuCl (present)	3.3	13.11
GuCl removed	95.0	4.94
GuCl removed in the presence of 25 mM β-ME	24.0	6.55
GuCl + 20 mM TCEP	2.7	13.23

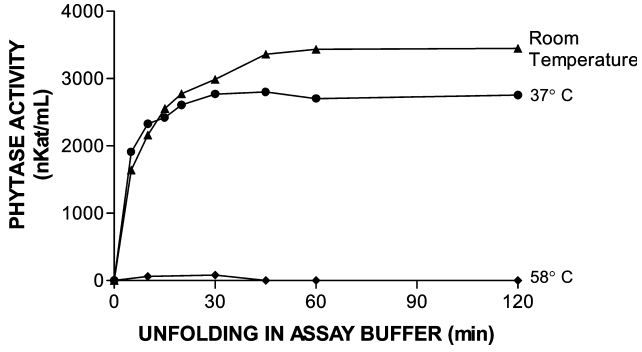

Fig. 3. Refolding of phytase as a function of time at 22, 37, and 58 °C.

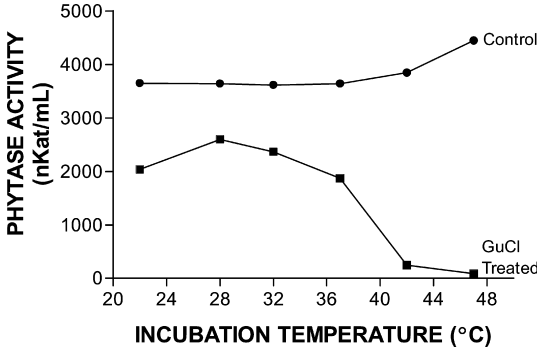

Fig. 4. Refolding of phytase at various temperatures.

Subsequent addition and removal of thiol reagent such as β-ME and TCEP had profoundly affected the refolding processes in phytase. The dynamic light scattering measurements of hydrodynamic radius of phytase under native, unfolded, and refolded state in the presence and absence of thiol reagent correlate strongly with the structure that gave activity. Thus, it was concluded that disulfide bridges play a crucial role in the folding of phytase.

The role of disulfide bonds in the conformational stability and catalytic activity of fungal phytase was studied in the presence of chemical denaturant, urea, and the kinetics of the unfolding process studied with or without thiol reagent, DTT [12]. Wang and co-worker's data dealing with intrinsic fluorescence spectra, far-ultraviolet circular dichroism (CD) spectra, and enzyme activity of denatured phytase had indicated that in the presence of 2 mM DTT, both inactivation and unfolding were greatly enhanced at the same concentration of the denaturant. The fluorescence emission maximum red shift and decrease of ellipticity at 222 nm were in full agreement with the changes of catalytic rate. The kinetics of unfolding processes, according to the study, were biphasic process consisting of two first-order reactions in the absence of DTT and a monophasic process in the presence of DTT. It was concluded that catalytic demise was most likely due to a conformational change in the active site and the disulfide bridges played a crucial role in the optimization of the three-dimensional structure of the active center of fungal phytase [12].

The results of unfolding and inactivation studies in phytase during thermal denaturation were reported recently [13]. Kinetics of inactivation and unfolding during thermal denaturation of phytase was compared for both phytate and synthetic substrate driven assays. The loss of phytase activity during thermal denaturation was found to be a reversible process. However, as for acid phosphatase activity, two phases of thermal inactivation were noticed; between 45 and 50 °C, the thermal inactivation was viewed as an irreversible process, which had some residual activity. Inactivation at temperature above 55 °C, no residual activity was observed. Fluorescence analyses have indicated that at temperature below 60 °C for 60 min, the conformation of the enzyme had no detectable change. However, above 60 °C, some fluorescence red shift could be observed with a decrease in emission intensity. All things considered, inactivation of phytase occurs before significant conformational changes of the protein. Thus, it appears that the active site of fungal phytase is more fragile than the overall conformation of the protein [13].

The knowledge-based structural improvement of fungal phytase through site-directed mutagenesis has caught the attention of researchers active in the field. The pH versus activity profile was already changed by this method [8]. A similar approach could be applied to understand the importance of disulfide bridges in phytase and engineer one more disulfide bridge in the catalytic site to impart stability so that thermal denaturation of the enzyme could be retarded at a temperature above 60 °C. This is an achievable goal, which could help the poultry feed industry to pelletize soybean meal mixed with active phytase.

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